INFECTION AND IMMUNITY, Aug. 1996, p. 3374–3378 Vol. 64, No. 8
0019-9567/96/$04.00+0

Functional Characterization of a Sialyltransferase-Deficient Mutant of *Neisseria gonorrhoeae*

MARTIN J. GILL,[1] DANIEL P. McQUILLEN,[2] JOS P. M. VAN PUTTEN,[3] LEE M. WETZLER,[2] JENNIFER BRAMLEY,[4] HELEN CROOKE,[4] NICHOLAS J. PARSONS,[4] JEFF A. COLE,[4] AND HARRY SMITH[5]*

Department of Infection,[1] Medical School,[5] and School of Biochemistry,[4] University of Birmingham, Birmingham B15 2TT, United Kingdom; Maxwell Finland Laboratory for Infectious Diseases, Boston City Hospital, Boston, Massachusetts 02118[2]; and Laboratory of Microbial Structure and Function, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, Montana 59840-2999[3]

Received 29 February 1996/Returned for modification 10 April 1996/Accepted 30 May 1996

Previous studies indicate that sialylation of lipopolysaccharide (LPS) by host CMP–*N*-acetylneuraminic acid (CMP-NANA) catalyzed by bacterial sialyltransferase rendered gonococci resistant to killing by phagocytes, to entry into epithelial cell lines, to killing by immune serum and complement, and to absorption of complement component C3. These results have been confirmed by comparing a sialyltransferase-deficient mutant (strain JB1) with its parent (strain F62) in appropriate tests. In contrast to F62, JB1 was very susceptible to killing by human polymorphonuclear phagocytes in opsonophagocytosis tests and incubation with CMP-NANA did not decrease the level of killing. The inherent resistance of F62 in these tests was probably due to LPS sialylation by CMP-NANA and lactate present in the phagocytes. A JB1 variant expressing the invasion-associated Opa protein was as able to enter Chang human conjunctiva epithelial cells as an Opa-positive variant of F62, suggesting that the sialyltransferase is not required for Opa-mediated entry. After incubation with CMP-NANA, the number of F62 variant gonococci entering cells but not that of JB1 variant gonococci was drastically reduced. Both JB1 and F62 were killed by incubation with rabbit antibody to gonococcal major outer membrane protein, protein I, and human complement, but only F62 was rendered resistant to the killing by incubation with CMP-NANA. Finally, both JB1 and F62 absorbed similar amounts of complement component C3 and the binding was decreased by incubation with CMP-NANA only for the wild type, F62.

Gonococci in vivo contain lipopolysaccharide (LPS) components with M_rs greater than 4.2 kDa and sialyl groups on terminal Galβ1-4GlcNAc epitopes of their carbohydrate chains (15). The sialyl groups are provided by host CMP–*N*-acetylneuraminic acid (CMP-NANA) (9, 15), and transfer is catalyzed by a gonococcal sialyltransferase (7) found in all strains examined (5). The sialylation is enhanced by another host product, lactate (10).

Many facets of gonococcal pathogenicity are affected by this sialylation of LPS by host CMP-NANA. It is responsible for the resistance of gonococci in urethral exudates to complement-mediated killing by human serum: indeed, its discovery stemmed from an investigation of why this resistance was lost on subculture (15). Also, complement-dependent killing by antibodies against gonococcal major outer membrane protein I (PI) was inhibited after gonococci were incubated with CMP-NANA (2, 20). Ingestion of gonococcal strains VPI and MS11 by human epithelial cell lines was inhibited by prior incubation of the gonococci with CMP-NANA (16). In opsonophagocytosis tests with human polymorphonuclear phagocytes (PMNs) and C8-depleted (CΔ8) serum, the survival rate of strain F62 was increased after incubation with CMP-NANA (4) and in nonopsonic tests (no antibody or complement), adherence and the oxidative burst were reduced and the survival time was longer (12). In several of these investigations, disturbances in the binding of complement components were detected (16, 20, 23). Finally, the results of some of these experiments fitted observations of the infectivity of gonococci in urethrally inoculated volunteers (14, 15).

Confirmation that sialylation of LPS by CMP-NANA is responsible for these aspects of pathogenicity would be obtained if the aspects were not manifested by sialyltransferase-deficient mutants containing LPS components capable of being sialylated. Recently, such a mutant (strain JB1) was obtained by insertion mutagenesis of strain F62 with transposon Tn*1545*-3 and screening for unlabelled colonies after incubating with CMP-[^{14}C]NANA (1). In contrast to those of the wild type, extracts of the mutant did not catalyze sialylation of LPS by CMP-NANA. It has been shown by Southern analysis that the mutant contains only a single copy of the transposon. Although there was a slight difference in LPS contents (see below), no other phenotypic differences between the mutant and the parent strain have been found. Nevertheless, until the disrupted locus has been cloned, sequenced, and shown to complement the mutant fully, the possibility that the mutation affects downstream genes of the operon by polarity cannot be excluded.

With regard to the difference in LPS contents, both the wild type and the mutant contained five LPS components with similar M_rs but the relative amounts of two components were different (1). As regards receptivity for sialylation, three LPS components with the same M_rs in both the wild type and the mutant contained sites for sialylation. They reacted with a monoclonal antibody (3FII) which detects the relevant epitope (Ga1β1-4G1cNAc). However, when the strains were incubated with CMP-NANA, only the components of the wild type became sialylated, as indicated by shifts in M_rs to higher levels and loss of reactivity with monoclonal antibody 3FII (1).

* Corresponding author. Mailing address: Medical School, University of Birmingham, Birmingham B15 2TT, United Kingdom. Phone: 0121-414-6920. Fax: 0121-414-5925.

In contrast to its parent strain, F62, the mutant JB1 was not converted to serum resistance by incubation with CMP-NANA, blood cell sonicates, or their diffusates (1). This finding confirmed that LPS sialylation is responsible for serum resistance. It also showed that blood cell extracts do not contain a system for sialylating gonococcal LPS which is independent of CMP-NANA, such as direct transfer of sialyl groups from glycoproteins (1). This paper describes comparisons of the mutant and the wild type in tests for (i) resistance to killing by PMNs in opsonophagocytosis tests, (ii) invasion of Chang human conjunctiva epithelial cell lines, (iii) inhibition of the bactericidal action of antibody to PI and complement, and (iv) disturbance of the action of complement and the effect of incubation with CMP-NANA on the behavior in these four tests of the two strains. In the experiments with Chang epithelial cells, we investigated not only the requirement of the sialyltransferase for CMP-NANA inhibition of entry but also the possible need for the sialyltransferase to achieve entry in the absence of CMP-NANA.

MATERIALS AND METHODS

Strains of *Neisseria gonorrhoeae*. Strain F62 was obtained from J. E. Heckels (Southampton General Hospital, Southampton, United Kingdom). Mutant JB1 was as described previously (1).

Opsonophagocytosis test. The opsonophagocytosis test method was similar to that used by others (4, 13).

Cells of strain F62 or JB1 grown overnight on agar as previously described (1) were suspended in a defined medium (DM) (19) at an optical density at 540 nm of 0.5 (ca. 10^8 CFU/ml). CMP-NANA (33 μM) was added to some samples of the suspension but not to controls. The suspensions were incubated for 1 h at 37°C with rotatory mixing.

PMNs were separated as follows from heparinized human peripheral blood (20 IU of heparin per ml) collected from a single healthy donor for each experiment. Heparinized blood (20 ml) was mixed with Hespan (3 ml; Dupont/Merck, Wilmington, Del.) and allowed to sediment at $1 \times g$ for 45 min at room temperature. The leukocyte-rich supernatant (7 ml) was layered onto Ficoll-Paque (8 ml; Pharmacia, Uppsala, Sweden) and centrifuged at $400 \times g$ for 20 min at room temperature. The PMNs in the pellet were resuspended in DM, and after the cell viability was determined by trypan blue exclusion, the suspension (>95% PMNs) was adjusted to 2×10^7 viable cells per ml.

The phagocytosis mixtures (250 μl) contained the following: 65 μl of the samples of gonococci (ca. 6×10^6 CFU) which had been incubated with or without CMP-NANA, 25 μl of CΔ8 human serum (Sigma, Poole, United Kingdom) with or without heat inactivation at 56°C for 30 min, 125 μl of the PMN suspension (ca. 2.5×10^6 PMNs), and 35 μl of DM. The mixtures were incubated at 37°C with rotatory mixing. Viable counts (CFU per milliliter) were made initially and after a 90-min incubation by plating triplicate serial dilutions and counting colonies after 24 h.

Test for adherence and invasion of Chang epithelial cells. The test for adherence and invasion of Chang epithelial cells was similar to that used in previous work (16).

Strains F62 and JB1 were routinely grown on GC agar medium (16, 20) for 16 h at 37°C in a 5% CO_2 atmosphere. For use in infection experiments, bacteria were grown (37°C) to mid-logarithmic phase in 10 ml of GC liquid medium (16, 20) in 50-ml Erlenmeyer flasks on a gyrotary shaker (125 rpm, 37°C) in the absence and presence of CMP-NANA (40 μM) as previously described (16).

Chang human conjunctiva epithelial cells (ATCC CCL 20.2) were maintained (37°C, 5% CO_2) in 5 ml of RPMI 1640 (GIBCO-BRL, Gaithersburg, Md.) supplemented with 5% fetal calf serum in 25-cm^2 tissue culture flasks (Corning Glass Works, Corning, N.Y.). For infection experiments, cells were grown on 12-mm-diameter circular glass coverslips in a 24-well tissue culture plate (1 ml of medium per well) for 48 h.

For infection experiments, bacteria were added to subconfluent epithelial cell monolayers maintained in RPMI 1640 at a bacterium/cell ratio of 50:1. When appropriate, CMP-NANA (10 μM) was added to the wells to assure maximum LPS sialylation. After a 2-h incubation at 37°C (5% CO_2), the cells were washed with Dulbecco's phosphate-buffered saline (PBS) to remove the nonadherent bacteria and fixed in PBS containing 1% paraformaldehyde and 0.1% glutaraldehyde (30 min, 24°C). Bacterial adherence and entry were scored by light microscopy as previously described (17, 18). Data are presented as the means ± the standard errors of five independent experiments. Data were analyzed by Student's *t* test for paired comparison. The ability or inability of the bacteria to incorporate sialic acid into their LPS was confirmed with CMP-[^{14}C]NANA (16).

The sialyltransferase activities of F62 and JB1 variants that were selected for expression of invasion-associated Opa protein were measured by a modification of a published method (7). Briefly, 5×10^8 gonococci suspended in 1 ml of PBS containing 0.5% Triton S-100 were disrupted by sonication (15 s, 4°C) and the supernatant was collected by centrifugation (20 min, $15{,}000 \times g$, 4°C). Ten microliters of the soluble bacterial extract was added to 90 μl of PBS (pH 6.8) containing 1 μM CMP-[^{14}C]NANA (150 mCi/mmol; Dupont, NEN, Boston, Mass.) as a sialyl substrate and 0.4 mg of asialofetuin (Sigma) as the sialyl acceptor. After 60 min at 37°C, the formed product was precipitated by addition of 100 μl of acetone (20 min, 4°C) and collected by centrifugation (20 min, $12{,}500 \times g$, 4°C). The pellet was washed once with acetone, and radioactivity was counted with a liquid scintillation counter (Beckman Instruments, Palo Alto, Calif.). Incubations without bacterial extract and with 0.4 mg of BSA instead of asialofetuin served as controls.

Bactericidal test using a mixture of rabbit antibody to PI and complement. PI was purified from a PIII-deficient strain of F62 and inserted into liposomes as previously described (21, 22). Two rabbits were immunized subcutaneously twice with a 2-week interval with 50 μg of PI in liposomes, and sera were obtained 2 weeks after the last immunization. Each rabbit developed a level of immunoglobulin G that elicited an enzyme-linked immunosorbent, half-maximum assay titer of greater than 1/10,000 (21). The serum from each rabbit was used separately in the bactericidal tests.

Gonococcal strains F62 and JB1 were grown overnight on GC agar plates in a 5% CO_2 incubator as previously described (20). Fresh gonococci were lifted from the plate with a dacron swab and suspended in GC liquid medium (1.5% [wt/vol] proteose peptone and 0.9% NaCl in 10 mM phosphate buffer at pH 7.2 with 1% IsoVitaleX enrichment [BBL, Cockeysville, Md.] to an optical density at 600 nm of 0.1 (ca. 10^8 organisms per ml). One milliliter of the suspension was added to 4 ml of GC liquid medium. CMP-NANA (8 μM; Sigma) was added to some tubes but not to others. The mixtures were rotated slowly for 3 h at 37°C and then diluted to an optical density at 600 nm of 0.1.

The bactericidal test was as described before (20). In brief, the reaction mixtures were as follows: 125 μl of gonococcal suspension, 25 μl of complement source, 25 μl of heat-inactivated antiserum (56°C, 30 min), and 75 μl of Hanks balanced salt solution (HBSS). The complement source was human serum adsorbed with glutaraldehyde-fixed gonococci to remove gonococcal antibodies (21). The mixtures were rotated for 1 h at 37°C. Aliquots were removed at the beginning and end of the incubation, diluted, and plated on GC agar for viable counts. Percent survival was calculated in relation to counts of samples incubated without complement (20).

Test for C3 binding. Normal human serum was obtained fresh from five adults with no history of neisseria infection, pooled, and stored at −70°C until use.

Human complement component C3 was purified by a modification of established methods (3). Briefly, fresh human plasma suspended in 0.06% EDTA was fractionated in 5% (wt/vol) polyethylene glycol 3350 (J. T. Baker, Inc., Phillipsburg, N.J.). The supernatant was brought to 16% (wt/vol) polyethylene glycol 3350 and then subjected to DEAE-Sephacel (Pharmacia, Inc., Piscataway, N.J.) chromatography. Fractions containing C3 were pooled. The C3 was labelled with ^{125}I to a specific activity of 6.6×10^4 cpm/μg with Iodobeads (Pierce Chemical Co., Rockford, Ill.) and then labelled C3 was separated from free ^{125}I by PD-10 (Pharmacia, Inc., Piscataway, N.J.) sieve chromatography.

Gonococci were grown overnight on plates in candle extinction jars at 37°C and then grown in a liquid medium (8) to exponential phase. For sialylation, 130 μM CMP-NANA was added to the growth medium. After two washes in gel-HBSS^{++} (Hanks balanced salt solution containing 1 mM $MgCl_2$, 0.15 mM $CaCl_2$, and 0.1% gelatin [Difco Laboratories, Detroit, Mich.]), the organisms were suspended (final concentration, 5×10^8/ml) in gel-HBSS^{++}.

The reaction mixtures (1 ml) consisted of 900 μl of the suspension of gonococci and 50 μl of pooled normal human serum supplemented with trace amounts of ^{125}I-C3. The mixtures were incubated at 37°C for 60 min with shaking, and then duplicate 400-μl aliquots were removed and immediately layered on top of 1,000 μl of ice-cold gel-HBSS^{++} and then centrifuged at $14{,}000 \times g$ (Beckman Microfuge II) at 4°C for 3 min. The pellets were resuspended in 500 μl of ice-cold gel-HBSS^{++} and then centrifuged again. Supernatants containing noncovalently associated ^{125}I-C3 were discarded. Gamma emissions from the pellets were counted in an LKB-Wallac 1282 Compugamma (Pharmacia LKB Nuclear Inc., Gaithersburg, Md.). Aliquots from the initial organism suspensions were plated for concomitant CFU determination. The number of C3 molecules bound per CFU was calculated by the following formula of cpm in pellet/cpm per molecule of ^{125}I-C3/CFU in pellet.

RESULTS

Strains F62 and JB1 were compared in the following four tests after being grown in the absence and presence of CMP-NANA.

Opsonophagocytosis tests with human PMNs. Opsonophagocytosis tests with human PMNs were done in the presence of human serum whose eighth component of complement had been depleted to ensure that any bactericidal effect was caused by opsonophagocytosis and not by complement-mediated extracellular processes. Controls demonstrated that the CΔ8 se-

TABLE 1. Comparison of strains F62 and JB1 in opsonophagocytosis tests with human PMNs after incubation without and with CMP-NANA

Expt	Strain	CΔ8 serum type	CMP-NANA	Opsonophagocytosis test result		*P* value[b]
				Viable count[a] (10^7) of CFU/ml		
				Initially[c]	After 90 min	
1	F62	Unheated	−	3.0 ± 0.8	1.0 ± 0.3	<0.02
	F62	Unheated	+	3.6 ± 1.1	2.8 ± 0.6	NS
	JB1	Unheated	−	1.5 ± 0.2	0.002 ± 0.0006	<0.001
	JB1	Unheated	+	1.5 ± 0.4	0.001 ± 0.0008	<0.01
	F62	Heated	−	3.3 ± 0.5	1.2 ± 0.8	<0.02
	F62	Heated	+	3.9 ± 0.6	3.6 ± 1.0	NS
	JB1	Heated	−	1.1 ± 0.3	0.7 ± 0.2	<0.05
	JB1	Heated	+	1.9 ± 0.4	0.1 ± 0.07	<0.001
2	F62	Unheated	−	11.0[d] ± *1.8*	4.8 ± 1.8	<0.02
	F62	Unheated	+	3.0 ± 0.9	2.8 ± 2.1	NS
	JB1	Unheated	−	4.5 ± 0.05	0.001 ± 0.0007	<0.001
	JB1	Unheated	+	3.7 ± 1.8	0.001 ± 0.0008	<0.001
	F62	Heated	−	9.2 ± 1.9	2.8 ± 0.6	<0.02
	F62	Heated	+	2.7 ± 0.8	3.7 ± 0.8	NS
	JB1	Heated	−	1.3 ± 0.1	0.23 ± 0.015	<0.001
	JB1	Heated	+	4.5 ± 1.8	0.16 ± 0.023	<0.05

[a] Means and standard deviations ($n = 3$).
[b] Twinned *t* test. NS, not significant.
[c] Except in one test, these viable counts did not differ by more than 50% from those made on the original culture before incubation with CMP-NANA (33 μM) in DM or with DM alone to provide the inocula for the tests. Also, control samples showed that counts of both F62 and JB1 were not significantly reduced by incubation for 90 min in unheated CΔ8 serum without addition of PMNs.
[d] In this test, a larger inoculum was inadvertently used for two of the samples.

rum was not bactericidal in the absence of PMNs. Tests were also carried out with heat-inactivated CΔ8 to see if nonopsonic phagocytic killing occurred and whether there were differences in levels of resistance between the strains.

In tests with unheated CΔ8, the mutant JB1 was far more susceptible to killing than the wild-type F62 and incubation with CMP-NANA did not prevent it from being killed (Table 1). Strain F62 was killed to some extent and its resistance was increased by incubation with CMP-NANA: thus, when strain F62 was incubated without CMP-NANA, viable counts after a 90-min incubation were significantly below initial counts, but this was not true for the gonococci incubated with CMP-NANA (Table 1).

When heat-inactivated CΔ8 was used, a low level of nonopsonic killing occurred with both strains (Table 1). Again, there was evidence for an increase in resistance of strain F62 to killing after incubation with CMP-NANA. Final counts of the organisms incubated without CMP-NANA were significantly below the initial counts, in contrast to the final counts of organisms treated with CMP-NANA (Table 1). No such evidence could be seen for the mutant JB1.

Adherence and entry into Chang human conjunctiva epithelial cells. First, nonpiliated variants of F62 and JB1 that expressed the invasion-associated Opa protein were selected by colonial morphology in conjunction with the [^{35}S]heparin sulfate proteoglycan receptor-binding assays (17). The presence or absence of sialyltransferase activity in the selected variants was confirmed. Infection of Chang cells with the selected F62 variant and the corresponding Opa-positive JB1 variant in the absence of CMP-NANA showed that both the wild type and the mutant strain efficiently adhered to and entered host cells (Fig. 1). Similar experiments using cells infected with gonococci in the presence of CMP-NANA (10 μM) demonstrated more than 85% inhibition of wild-type F62 entry into the host cells with a moderate decrease (50%) in bacterial adherence (Fig. 1). In contrast, strain JB1, which is defective in sialyltransferase activity, adhered to and entered the Chang cells at levels similar to those of strain F62, irrespective of the presence of CMP-NANA (Fig. 1).

Killing by anti-PI rabbit serum and complement. Almost all the wild-type and mutant bacteria were killed after a 1-h incubation at 37°C with a mixture of anti-PI rabbit serum and complement (Table 2). As observed previously for strains Pgh 3-2 and UUI (20), strain F62 became fully resistant after incubation for 3 h with CMP-NANA (8 μM). In contrast, the mutant JB1 remained fully susceptible after such treatment.

Absorption of complement component C3. Strains F62 and JB1 bound similar amounts of C3. However, growth of the former with CMP-NANA decreased C3 binding by 40% ($P < 0.002$) but binding to the latter was not changed by this treatment (Fig. 2).

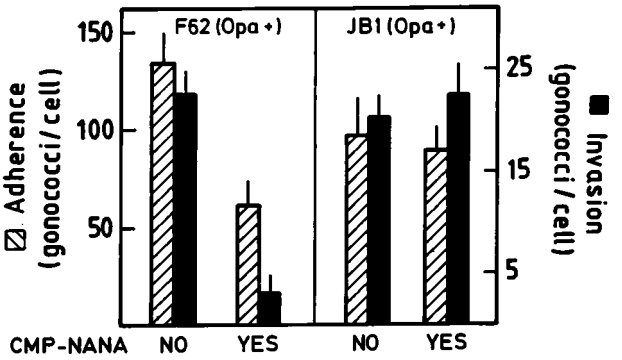

FIG. 1. Adherence and entry of Opa-positive variants of strains F62 and JB1 into Chang epithelial cells in the absence and presence of CMP-NANA (1 h of infection). Values are the means ± the standard errors of five experiments. Significant *P* values: adherence of F62 versus that of F62 with CMP-NANA, <0.05; invasion of F62 versus that of F62 with CMP-NANA, <0.005.

TABLE 2. Action of a mixture of anti-PI rabbit serum and complement on strains F62 and JB1 after incubation without and with CMP-NANA

Serum of rabbit	Strain	CMP-NANA	% Survival[a] in each of three experiments
1	F62	−	<5
	F62	+	>95
	JB1	−	<5
	JB1	+	<5
2	F62	−	<5
	F62	+	>95
	JB1	−	<5
	JB1	+	<5

[a] Percent survival was calculated in relation to the counts of control samples incubated without complement.

DISCUSSION

The results of the opsonophagocytosis tests were dramatic, not so much for the effect of CMP-NANA treatment on the two strains as for the different behaviors of the untreated organisms. The extreme susceptibility of JB1 compared with the relative resistance of F62 (Table 1) means that LPS sialylation is of paramount importance in resisting the killing action of phagocytes. This resistance of F62 could be due to either resisting ingestion by phagocytes or prevention of intracellular killing or both. Human PMNs contain both CMP-NANA and lactate (9, 10, 11, 15), and in previous phagocytosis experiments with gonococci and human PMNs, sialylation of gonococcal LPS occurred within 10 min (6). The relative resistance of untreated F62 to opsonophagocytic killing (Table 1) is probably due to its use of the CMP-NANA and lactate that become available from the PMNs during the phagocytosis tests. Against this background, the effect of prior incubation with CMP-NANA would be expected to be nil for the mutant and small for the wild type. This happened (Table 1), and the effect of CMP-NANA on the survival of F62 was significant but not dramatic. Thus, in the tests with unheated CΔ8, mean survival rates at 90 min of 33 and 44% increased to 77 and 93% for the organisms treated with CMP-NANA, respectively, in experiments 1 and 2 (Table 1). In previously described opsonophagocytosis tests (4), a different sample of strain F62 was more susceptible to killing than the one used in the tests reported here; gonococcal survival rates were 25, 10, and 4% after 30, 45, and 60 min of incubation with phagocytes, respectively. After incubating the gonococci with CMP-NANA, these survival rates increased to 51, 20, and 15%, respectively. Consistent with the above discussion, these increases were significant but again not dramatic despite a longer incubation with a higher concentration of CMP-NANA than reported here (4). Further experiments are required to determine whether sialylation exerts its main protective effect on ingestion or intracellular killing by phagocytes.

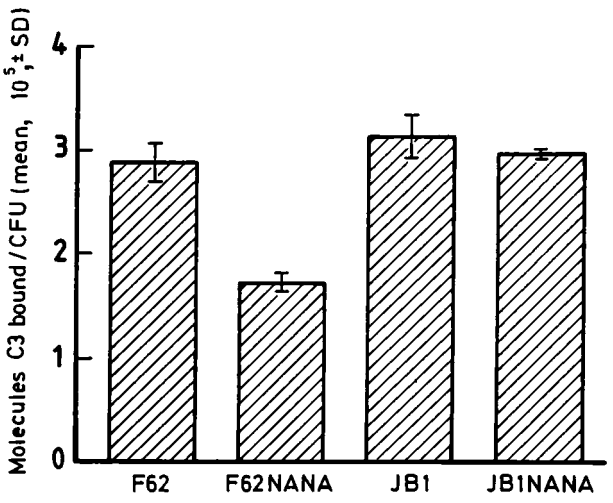

FIG. 2. C3 binding to the wild type and the mutant after incubation without (bars labelled F62 and JB1) and with CMP-NANA (bars labelled F62 NANA and JB1NANA). For measurements of the number of molecules of C3 bound, see Materials and Methods. These results are representative of those from two experiments. SD, standard deviation.

There were two important results from the experiments with Chang epithelial cells. First, the fact that the mutant and the wild type had similar capacities to enter these cells shows that sialyltransferase is not required for the entry process. It is possible, for example, that the enzyme transferred cell surface sialyl groups to the bacteria, thereby unmasking previously unexposed receptors for opacity proteins. Second, incubation with CMP-NANA did not abrogate entry of the mutant JB1 into Chang epithelial cells, in contrast to its inhibitory effect on wild-type invasion (Fig. 1). These data support the view that LPS sialylation decreases the level of invasive behavior of gonococci towards this cell type.

The results of the bactericidal tests and those for C3 binding were not unexpected. The complete conversion of F62 from showing susceptibility to showing resistance to the combined action of PI antibody and complement by incubation with CMP-NANA without any sign of a similar effect on the susceptibility of the mutant JB1 was satisfying (Table 2). Previous work with strains Pgh 2-3 and UU1 suggested that the resistance endowed by CMP-NANA in this system can be attributed to a negative effect of sialyl groups on the complement system rather than prevention of antibody binding (20). This explanation fits the fact that the level of binding of C3 to the wild type is decreased by incubating it with CMP-NANA, in contrast to that to the mutant (Fig. 1). Also, the effect of sialylation on C3 binding, which has been noted before (23), may have influenced the opsonophagocytosis tests, since C3 binding is important in both humoral and phagocytic defense.

Clearly, the properties of the mutant JB1 confirm the importance of sialylation of gonococcal LPS in resistance to killing by phagocytes, in stopping epithelial cell invasion, in preventing the bactericidal action of immune serum and complement, and in disturbing absorption of C3, which can affect both humoral and cellular defenses.

ACKNOWLEDGMENTS

N. J. Parsons and part of the work at the University of Birmingham, Birmingham, United Kingdom, were supported by the Wellcome Trust (reference no. 036791/2/92/Z/1.5/PMC/RF). D. P. McQuillen's work is supported by a grant (reference no. K11 A101061) from the National Institutes of Health (National Institute of Allergy and Infectious Diseases).

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Editor: T. R. Kozel